Gene xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

An androgen-regulated miR-2909 modulates TGFβ signalling through AR/miR-2909 axis in prostate cancer

Shiekh Gazalla Ayub[a,*], Deepak Kaul[a], Taha Ayub[b]

[a] Department of Experimental Medicine and Biotechnology, Post-Graduate Institute of Medical Sciences and Research, Chandigarh, India
[b] Department of SPM, Govt. Medical College, Srinagar, India

ARTICLE INFO

Keywords:
Prostate cancer
Hormone-sensitive
Castration-resistant
Androgen receptor

ABSTRACT

In recent years, microRNAs (miRNAs) have emerged as promising biomarkers for PCa diagnosis and prognosis. miR-2909 is a novel miRNA that can regulate immunogenomics and oncogenomics. The present study investigated the role of miR-2909 in the pathogenesis of PCa and the potential signalling pathways through which it operates. We have identified miR-2909 as a novel mediator of androgen/androgen receptor (AR) signalling that enhances the proliferation potential of PCa cells and assists in cancer survival under reduced androgen levels. Our results revealed that miR-2909 down regulates TGFBR2 by targeting its 3′-UTR sequence. We also observed that miR-2909 over-expression attenuated TGFβ-mediated SMAD3 activation, cell growth inhibition and apoptosis. Moreover, miR-2909 modulated the expression of p21CIP, c-MYC and CCND1 through TGFβ signalling. Importantly, we also demonstrated that miR-2909 and AR regulates each other's expression resulting in a positive feedback loop. In conclusion, our study suggests that miR-2909 is an androgen-inducible miRNA that exerts its oncogenic effects by attenuating the tumor-suppressive effects of TGFβ signalling.

1. Introduction

PCa is considered the second leading cause of cancer death in men older than 40 years worldwide (Ferlay et al., 2015). Androgen deprivation therapy (ADT) represents the most effective therapy for patients with hormone-sensitive PCa (HSPC), (Loblaw et al., 2007) however, many patients invariably relapse with more aggressive form, which has limited therapeutic options and poor prognosis (Pienta and Bradley, 2006). A current challenge in PCa management is to understand the molecular mechanisms controlling disease progression. In this context, miRNAs have assumed importance and have recently become an attractive area of research. miRNAs function as oncogenes or tumor-suppressor genes, (Heneghan et al., 2010) and their deregulation is a common feature of human cancers including PCa (Ayub et al., 2015). Recent findings from our lab have shown that apoptosis antagonizing transcription factor (AATF) gene encodes a miRNA designated as miR-2909 which regulates various important genes involved in different pathological conditions including cancer (Malik et al., 2014; Arora et al., 2014). As AATF is known co-activator of AR through its interaction via LXXLL motifs, it could be assumed that miR-2909 plays a vital role in PCa pathogenesis.

AR signalling known to play a critical role in the development of normal prostate, (Wen et al., 2015) is modified/deregulated to promote cell survival and proliferation in PCa (Lonergan and Tindall, 2011). One of the important pathway AR cross-talks with is TGFβ signalling (Cao and Kyprianou, 2015; Zhu and Kyprianou, 2008). Multiple studies have shown that TGFβ-induced inhibitory effects are blocked by AR signalling to promote growth and proliferation of PCa cells (Hayes et al., 2001; Lucia et al., 1998). However, the exact molecular mechanism of this crosstalk between AR and TGFβ signalling has not been completely understood.

In this study, we have identified miR-2909 as an androgen-regulated miRNA and have further investigated its mechanism of action. To the best of our knowledge, this is the first study to show that AR and miR-2909 drives the down-regulation of TGFBR2 by acting through positive feedback loop and thereby attenuating the inhibitory effects of TGFβ signalling.

2. Materials and methods

2.1. Cell culture

PC3 and LNCaP cells were obtained from NCCS, India. The cells

Abbreviations: TGFβ receptor II, TGFBR2; Prostate cancer, PCa; Androgen deprivation therapy, ADT; Hormone-sensitive PCa, HSPC; Apoptosis antagonizing transcription factor, AATF; Androgen receptor, AR; Dihydrotestosterone, DHT; PSA, Prostate Specific Antigen; Estrogen receptor-b, ERb; AD, Androgen dependent; AI, Androgen independent; GFP, Green fluorescent protein; ADT, Androgen deprivation therapy

* Corresponding author.
E-mail address: medwrit@vmrcindia.com (S.G. Ayub).

http://dx.doi.org/10.1016/j.gene.2017.07.037
Received 9 April 2017; Received in revised form 8 July 2017; Accepted 11 July 2017

were cultured in medium recommended and maintained at 37 °C. For androgen treatment, cells were treated with 10 nM dihydrotesterone (DHT).

2.2. RNA Extraction, cDNA synthesis and qRT-PCR

Total RNA was isolated using miRNeasy kit (Qiagen) and reverse-transcribed to make cDNA using miScript Reverse transcription kit (Qiagen). After cDNA synthesis, Quantitative RT-PCR was performed using miScript-SYBR Green Mix (Qiagen) and RT-PCR (Stratagene). β-Actin and U6B were used as invariant controls for mRNA and miRNA quantification.

2.3. Western blot analysis

Total cellular protein was extracted using Laemmli's buffer and the protein levels were determined through western blotting using appropriate antibodies. β-actin antibody was used as an internal control. The protein concentration was determined by Bradford method and equal concentration of protein (g/ml) was loaded.

AR antibody is from Cell signalling, TGFBR2 (Biorbyt), T-SMAD3 and p-SMAD3 (abcam), and antibodies to Actin, Histone-H3, c-MYC, $p21^{CIP}$ and CCND1 are from Sigma.

2.4. Plasmid constructs and reporter assays

Primers were designed to amplify the 3′-UTR sequence of human TGFBR2 gene, and BamH1 sequence was added to the primer-ends. Cloning of 3′-UTR was performed in miRNASelect™ pMIR-GFP-reporter vector (Cell-Biolabs, USA) according to manufacturer's instruction. The plasmid constructs were co-transfected with miR-2909 along with or without anti-miR-2909 inhibitor (EXIQON). After 48 h, FACS analysis was performed to quantitate the number of cells expressing GFP.

For promoter analysis, promoter sequence of prostate specific antigen (PSA) with putative AR binding site was cloned into pBlue-TOPO reporter vector. For analysis of PSA transcriptional activity, the β-gal constructs were co-transfected with miR-2909 mimic or anti-miR-2909 inhibitor and β-galactosidase activity was measured after 48 h. Lentiviral cloning and expression vector with miR-2909 precursor sequence was used for miR-2909 over-expression and Control null vector and miScript Inhibitor NC (EXIQON) were used as respective controls. All transfections were performed with Escort transfection reagent (Sigma).

2.5. Cell cycle analysis

Cell cycle analysis was done on PC3 cells transfected with miR-2909 and LNCaP grown in androgen-deprived or complete media and transfected with miR-2909 or antagomiR-2909 (50 nM) and miScript-Inhibitor-NC (50 nM) for 48 h. Cells were fixed in 70% ethanol and labelled with PI. Percentage of cells in different phases were analysed with FACS Calibur cytometer (Becton Dickinson, USA).

2.6. Apoptosis assay

PC3 cells were transfected with miR-2909 mimic (20 nM) and Control null-vector (NC) for 48 h. Cells were then replated and treated with TGFβ1 (2 ng/ml) in complete medium for 5 days. Cells were trypsinized and labelled with PI and cell death was assayed by FACS Calibur cytometer.

2.7. Cell proliferation ELISA assay

PCa cells were plated in 96-well plate at 5×10^3cells/well in triplicates and grown in media supplemented with or without androgens. After 24 h, cells were transfected with either miR-2909 or anti-miR-2909 and their respective controls and grown for 48 h. Cell proliferation was quantified at 460 nm wavelength on a microplate reader (Tecan) using cell proliferation assay kit (Chemicon, Millipore).

2.8. Statistical analysis

Statistical analyses were performed by SPSS windows version19. Anova test was used to analyse groups of different data sets followed by appropriate post-hoc test. Data were presented as mean ± SD of experiments done in triplicate. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. miR-2909 is an androgen-inducible miRNA and AR signalling is essential for androgen-mediated regulation of miR-2909

Comparison of AR-negative and AR-positive PCa cell lines using PC3 and LNCaP respectively, a significantly high expression of miR-2909 was observed in LNCaP cells (Fig. 1A). Further, miR-2909 expression decreased significantly in LNCaP cells grown in androgen-deprived media (RPMI + charcoal-stripped FBS) compared with the control cells grown in complete media (RPMI + FCS) over a period of 24 h (Fig. 1B). This observation was significantly reversed upon addition of DHT and the miR-2909 expression was comparable to levels observed in cells grown in complete media (Fig. 1C). The miR-2909 expression also increased proportionately when LNCaP cells were treated with different concentrations of DHT ranging from 0.1–10 nM and analysed after 24 h (Fig. 1D). The efficiency of androgen-treatment was verified by PSA and AR mRNA up regulation.

Estrogen receptor-b (ERb) signalling can be cross-activated by androgens. To determine that the DHT-mediated up regulation of miR-2909 is mediated through AR and not ERb, stimulation of LNCaP cells with DHT for 48 h in presence or absence of AR antagonist, bicalutamide (Fig. 1E) and si-AR led to significant blockage of DHT-induced miR-2909 expression. The efficiency of bicalutamide was proven by the inhibition of DHT-mediated up-regulation of PSA (mRNA) and si-AR was proven by western blotting (Fig. 1F). The dependence on AR signalling was further confirmed by unaltered miR-2909 expression in AR-negative PC3 cells stimulated with different concentrations of DHT for 48 h (Fig. 1F).

3.2. miR-2909 stimulates androgen-dependent (AD) and androgen-independent (AI) growth

As AR signalling plays a vital role in PCa development, we investigated if miR-2909 plays any role in androgen-induced or androgen-independent cell growth using WST-1 cell proliferation assay. Ectopic expression of miR-2909 enhanced the growth of LNCaP cells in complete media as well as androgen-depleted conditions (Fig. 2A). To further establish therole of miR-2909 in cellular proliferation, bicalutamide treatment of miR-2909-over-expressing LNCaP cells whether grown in presence of normal physiological androgen levels or androgen-depleted conditions was found to inhibit the cell proliferation rate although not comparable to LNCaP-NC-treated cells which exhibited around 70% inhibition (Fig. 2A). Similarly, anti-miR-2909 treatment inhibited the growth of AD LNCaP cells grown in normal physiological androgen levels, but no significant inhibition was observed in LNCaP cells treated with anti-miR-2909 in androgen-deprived conditions (Fig. 2A). Interestingly, overexpression of miR-2909 significantly stimulated the growth of PC3 cells also (Fig. 2B).·Cell cycle assay by DNA flow cytometry also showed that ectopic expression of miR-2909 almost doubled S-phase fraction in LNCaP cells (Fig. 2C) and increased by about 54% in PC3 cells (Fig. 2D), whereas anti-miR-2909 treatment resulted in 60% reduction of S-phase in LNCaP cells (Fig. 2E).Taken together, these results demonstrate that miR-2909 is associated with both AD and AI growth of PCa cells.